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*Review*

# Galactolipase and chilling sensitivity of plants*

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**Key words**: chilling stress, chloroplasts, free fatty acids, galactolipase, galactolipids, lipid degradation, maize seedlings, phospholipids

**Galactolipase is a lipid acyl hydrolase (EC 3.1.1.26) acting predominantly on galactolipids which constitute up to 80% of total acyl lipids in chloroplast membrane.**

**Evidence is presented on the involvement of this enzyme in plant response to chilling *via* degradation of membrane lipids and the increase of free fatty acids, associated with reduced oxygen evolution in the Hill reaction. The occurrence of two pools of fatty acids has been hypothesized. Analysis of numerous plant species showed higher galactolipase activity in the chilling-sensitive than in the chilling-resistant plants. Differences in the pH-dependence curve and in the response to detergents of galactolipases from these two groups of plants suggest heterogeneity of the enzyme.**

**Referring to the hypothesis concerning the role of high melting-point fatty acids of phosphatidylglicerol molecular species in chilling sensitivity the data are presented against generalization of this hypothesis.**

## CHILLING STRESS AND CHILLING INJURY

Chilling sensitivity appears in plants when germination, growth, development of reproductive organs and storage are restricted to a temperature range from 0°C to even about 15°C in the case of tropical plants. Certain stages of the plant life cycle are more sensitive to chilling than others. The term "chilling injury" refers to the visual manifestation of cellular dysfunction of plants exposed to chilling temperatures within the temperature range defined for a given species. The symptoms of chilling injury vary depending on the type of tissue, its state of maturity and metabolic status of a plant (active or dormant) and on a variety of environmental factors. Visible symptoms of injury concern surface pitting, necrotic areas and external discoloration resulting from disruption of normal metabolic processes and degradation initiated or accelerated by chilling [1–3].

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**Abbreviations**: CS, chilling sensitive; CT, chilling tolerant; CR, chilling-resistant; FA, fatty acid; FFA, free fatty acid; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; SQDG, sulfoquinovosyldiacylglycerol; Chl, chlorophyll; $L_\alpha$-to $L_\beta$, liquid-crystalline phase to gel phase; LAH, galactolipase, lipid acyl hydrolase EC 3.1.1.26.

In the overall processes of chilling injury two stages should be considered: a primary event and a series of secondary events appearing in consequence of the primary event [4]. The primary event might involve a change in membrane lipid structure, conformational changes of regulatory enzymes and structural proteins [5] or an alteration of cytoskeletal structure. A primary event is more or less instantaneous, it occurs at a critical temperature at the onset of chilling injury and is reversible. The secondary events include the metabolic and ionic imbalances, the loss of cellular integrity or other events that lead to visible symptoms of injury which usually develop more rapidly when a plant is transferred to a non-chilling temperature. The secondary events are both time and temperature dependent. In short term it could be reversible if the stress is removed, but becomes irreversible when the stress is prolonged. The main problem in the research on chilling injury is to distinguish between the temperature dependent "cause" (primary event) which initiates the injury process from the time dependent effect (secondary events) [4]. Thus, according to Raison & Lyons [6] and Raison and Orr [4] susceptibility to chilling refers to "the temperature below which injury develops, i.e., the temperature of the primary event."

Chilling sensitive plants are those which suffer damage at chilling temperature beyond a certain limited time and, if maintained at this temperature, develop injuries leading to death. Chilling insensitive plants continue to grow and develop, but can not complete their life cycle at chilling temperatures near 0°C. Tolerance and resistance to chilling should be considered in terms of the response of a chilling sensitive plant and refers to the time course of the secondary effects. According to Raison & Orr [4] "the terms resistant or tolerant should be used in a quantitative and comparative sense to imply differences in plant behaviour under defined conditions". Consequently it has been also plainly defined that "if a plant is insensitive to chilling it does not suffer stress at chilling temperatures and, therefore, is neither resisting (CR) nor tolerating (CT) the chilling".

Possible mechanisms of chilling injury were recently discussed by Parkin *et al.* [7] and Saltveit & Morris [8].

## MEMBRANE LIPIDS IN CHILLING STRESS

In 1973 Lyons [1] and Raison [2] suggested that the primary event of chilling sensitivity is an $L_\alpha$-to-$L_\beta$ lipid phase transition in the cellular membranes. According to this proposal, transition from liquid crystalline phase to gel phase depends on the proportion of unsaturated fatty acids and would result in alterations of the metabolism of chilled cells, leading to injury and death of the chilling sensitive plants. Ten years later Murata *et al.* [9, 10] referred this hypothesis to chloroplast membrane and found a positive correlation between chilling sensitivity of plants and the level of saturated and *trans*-monosaturated molecular species of phosphatidylglycerol (PG) in thylakoid membranes. A downward shift in growth temperature generally increases the degree of unsaturation of membrane lipids, which compensates for the decrease in membrane fluidity brought about by the downward shift in temperature. Thus unsaturation of membrane lipids is considered to be one of the most critical parameters for functioning of biological membranes and, therefore, for the survival of organisms at lower temperatures [11]. In about 20 plant species it has been found that the content of disaturated PG in CR plants is lower, ranging from 3% to 19% of total PG than in CS plants in which disaturated PG constitute 26%–65% of total PG [12]. The discussed hypothesis assumes that the molecular species of chloroplast PG containing a combination of saturated fatty acids (FA) (16:0, 18:0, 16:1-*trans*) at both *sn*-1 and *sn*-2 positions of glycerol backbone confers chilling sensitivity of plants. PG species containing 16:1-*trans* 16:0 or 18:0 FA have been called "high-melting-point" (HMFA) PG molecular species [13], or disaturated PG molecular species [14]. The *trans*-monosaturated species (16:1-*trans*) are considered saturated [15], since the *trans*-double bond does not decrease the phase transition tem-

peratures as much as does the *cis*-double bond.

The hypothesis on the role of unsaturated FA in PG molecular species was supported by detection of $L_\alpha$-to-$L_\beta$ phase transitions in synthetic 16:0/16:0 PG molecular species and also in the PG isolated from CS plants, that were not observed in PG from CR plants [16]. In addition, phase transitions were detected by differential scanning calorimetry in the total lipids from the leaves [17] and thylakoids [18] of CS plants.

Recently it was demonstrated that genetic manipulation of FA saturation in PG can alter plant chilling sensitivity. Murata *et al.* [19] transformed tobacco plants introducing glycerol-3-phosphate acyltransferase cDNA from squash, a CS plant, or from *Arabidopsis*, a CR plant. When the squash enzyme was introduced, the content of saturated FA was increased in PG with a concomitant increase in chilling sensitivity. Introduction of the *Arabidopsis* enzyme decreased the content of saturated FA in PG and reduced chilling sensitivity. Wolter *et al.* [20] transformed *Arabidopsis* with a modified version of the *Escherichia coli* gene that encodes glycerol-3-phosphate acyltransferase. The transgenic plants showed an increase of 16:0 FA in the membrane lipids, in particular the fraction of high melting point fatty acids in PG, and were more susceptible to chilling than were wild-type *Arabidopsis*.

Although the deleterious effects of HMFA in PG seem to be indisputable [21], the question has been posed to what extent the presence of disaturated FA in PG is the direct and the sole reason of chilling sensitivity. Could other cellular targets for chilling injury mask the beneficial effects of the genetically modified level of disaturated FA [22]?

## DOES THE DISATURATED PG HYPOTHESIS EXPLAIN CHILLING SENSITIVITY OF PLANT CELLS ?

In the list of CS and CR plants, extended over the list of Murata *et al.* [9, 10] by Roughan [14] and Kenrick & Bishop [13]. A number of interesting exceptions to the general correlation was noted by Bishop [23] between the content of disaturated PG and chilling sensitivity within a genus. The differences in the content of HMFA in PG are small (50%–60%) among 14 species of *Solanaceae* despite large differences in their chilling sensitivity.

The HMFA level above 70% was found in CS cucumber, runner bean and *Amaranthus powelli* [14]. Similarly the content of disaturated FA in PG in CR pea and CS tomato is the same, i.e. 58%–60% and 55%–60% [23], respectively.

As the total PG content in thylakoids of most plants is approximately 10 mol% the total level of disaturated PG varies from 0.6% to 6.5% and from 0 to 4.2% in CS and CR plants, respectively [24]. These values are overlapping and do not distinguish these two groups. This led to the conclusion that the level of HMFA in PG is rather related to the genetic origin of a plant than to the degree of chilling sensitivity [23].

The analysis of fatty acid composition of PG in closely related species indicates that the content of HMFA *per se* does not seem to be an appropriate criterion for establishing the relationship between PG and chilling sensitivity. This holds for 17 varieties of rice (*Oryza sativa*) [25] and also for the closely related populations of black mangrove (*Avicennia germinans* L.) [26]. Similar levels of disaturated PG, 61%–65% were found in CR *Passiflora edulis* Sims. [13, 14] and 65% in CS *Passiflora edulis* Sims. *f. flavicarpa* Deg. [13].

*Arabidopsis fab*1 mutant may be considered "rebelious". As reported by Wu & Brose [27], the leaf PG of this mutant contains 43% of HMFA (*vs* about 9.9% in wild type *Arabidopsis*), i.e. more than in many chilling sensitive plants, however it is completely unaffected by lowering the temperature to the level which rapidly leads to death of other chilling sensitive plants.

Association of lipid phase transition with the content of HMFA-PG is not obvious either. Composition of the thylakoid lipid was altered, as expected, on acclimation of oleander clone [28] and cotton [29] to a lower temperature, but in cucumber these temperature-dependent changes were not observed [29] and growth at lower tempera-

ture resulted only in lowering of the total amount of PG without any change in proportion of the saturated PG molecular species [30].

Spin label measurements of CS *Passiflora flavicarpa* leaf polar lipids [31] indicated phase transition at 9°C, i.e. about 6°C above that of the closely related species, but more than that found in CT *P. edulis*. In spite of these differences, both species have an identical level of HMFA in their PG and in *Passiflora* species tested the correlation between the phase transition temperature of polar lipids and ion leakage from chilled leaves was not reflected in HMFA levels of leaf PG [31].

The effect of composition of PG from thylakoid polar lipids on the transition temperature was studied by Murata & Yamaya [16] and Raison's group [17, 18, 32, 33]. Addition of only 1 mol% of dipalmitoylphosphatidylglycerol to wheat tylakoid polar lipids triggered the onset of a $L_\alpha$-to-$L_\beta$ phase separation at 10°C [17]. This, however, does not mean that the transition temperature of polar lipids could be predicted from the total sum of the saturated FA or disaturated PG molecular species, since the polar lipid transition appears to be a product of combined effect of both high and low melting point lipids [32, 33].

Recently Webb *et al.* [24] have presented data showing that in well-defined large unilammelar vesicles composed of lipid mixtures similar to those of the thylakoids of CS and CR plants, the $L_\alpha$-to-$L_\beta$ phase separation does not occur between 0°C and 60°C. It was concluded, therefore, that it is unlikely that disaturated PG play a direct role in chilling injury by increasing permeability of the thylakoid membrane at low temperature. Other authors express objections to the possible abrupt phase transition above 0°C in the bilayer of native plant membranes composed of a heterogeneous mixture of membrane lipids containing predominantly unsaturated fatty acids [34, 35]. A question seems to be still unresolved in spite of more than 10 years of studies: could such minor changes in the molecular ordering of PG in membrane lipids have so many effects on cellular function [36]?

Contrary to the investigations on membrane lipids [12] in chilling stress much less attention has been paid to the enzymes degrading lipids. One of them, galactosidase, may be one of the factors important in development of primary event of chilling stress. This factor should be taken into consideration following the suggestion of Thomas *et al.* [37], that changes in one lipid can result in a massive reorganization of membrane constituents. It may be expected that such changes in membrane lipids will affect its fluidity and, therefore, its response to chilling stress.

## GALACTOLIPASE

The first information on the enzyme hydrolyzing unsaturated galactolipids was that of Sastry & Kates in 1964 [38] who detected its activity in the extracts of primary leaves of *Phaseolus multiflorus*. According to those authors a partially purified enzyme was specific for unsaturated galactolipids and was subsequently named galactolipase, but lately it has been classified as a non specific lipid-acyl hydrolase [39] (galactolipase EC 3.1.1.26). Except for the report of Burns *et al.* [40] who have separated two distinct hydrolases from the leaves of *Ph. multiflorus,* one with galactolipase activity and another showing phospholipase activity, in all other experiments reported a single non specific enzyme has been isolated acting both on galactolipids and phospholipids.

Galactolipase and galactolipids are localized mainly for chloroplast membranes [41] where galactolipids constitute from 70% to 80% of total acyl lipids. According to O'Sullivan *et al.* [42] in wheat thylakoids the enzyme is localized at the stromal surface of thylakoid membrane.

All the lipolytic acyl hydrolases isolated so far from the leaves hydrolyse galactolipids into two molecules of FFA and one mono- or digalactosylglycerol [38, 39, 43]. Neither exogenous FFA nor those released by galactolipase do inhibit the enzyme activity [39]. Conversly FFA exhibit stimulatory effect [39].

Quantitative differences in specificity of galactolipase reported depended on the kind of prepartion examined by the authors: the isolated, purified enzyme, chloroplast parti-

cles, or thylakoid membranes. For the cowpea enzyme the following affinities were reported in the decreasing order: DGDG, MGDG, PC, PG [44]. In wheat thylakoids the affinity towards DGDG was also higher than towards MGDG [42], however the reverse relation was found in spinach [45]. When spinach sub-chloroplast particles [41] or bean thylakoids [46] were digested by bean galactolipase, practically only galactolipids were hydrolysed, since MGDG and DGDG constituted 55% and 26% of total lipids and PG, PC, SQDG only 7.5%, 5.7% and 4.5%, respectively [46]. Similar proportions were found during ageing of wheat thylakoids [42].

**pH optima**

Various pH optima were reported for the purified galactolipase with its main substrates: ranging from 5.0 to 7.5 for MGDG [40, 41, 43, 44, 47, 48] and from 4.3 to 6.5 for DGDG [40, 41, 44, 47, 48, 49]. When spinach chloroplast particles [41] or thylakoid membranes [50–53] were used as substrates of bean galactolipase, the optimum pH was 7.0 since it was close to that for MGDG, which predominates in chloroplasts. In wheat chloroplasts the pH optima for MGDG and DGDG hydrolysis were 6.0 and 6.2, respectively [42].

**Effect of temperature**

Both degradation of galactolipids in detached tomato leaves [50] and tomato fruit pericarp [54, 55] and accumulation of FFA [56–60] during chilling stress at 0–4°C indicated that at low temperature the enzyme activity was not diminished. Similarly, no essential difference in the enzymatic activity and the release of FFA at 0° or 20°C were observed in wheat thylakoids [42]. Also the enzyme in tomato fruit microsomes maintained its activity when the temperature was lowered from 22° to 0°C [54].

Freezing damage resulting in cell disruption and release of degradative enzymes including galactolipase was found to be associated with almost total loss of galactolipids in potato leaves subjected to –4°C [61]. Chilling does not presumably result in damage of cell and chloroplast membranes [62, 63] to the extent which would stimulate galactolipase.

**Relative molecular mass and isoelectric point**

The relative molecular masses reported for galactolipase from different sources varied from 60000 to 110000 [41, 47] even for the same *Phaseolus* species the reported values were different: 60000 [43], 80000 [49] and 90000 [64]. In *P. multiflorus* $M_r$ of the enzyme was reported to be 110000, while that from *Vigna unquiculata* 80000 [44] and from potato 110000 [47].

Generally lipid acyl hydrolases are acidic proteins with pI values ranging from 4.4 [64], 4.6 [47], 5.0 [44] up to 7.0 [45].

**Effect of detergents**

The reported effects of detergents on galactolipase activity of both isolated preparations and intrinsic chloroplast enzyme are diverse. Thus, in each of the galactolipase assay systems either an inhibitory or a stimulatory effect of particular detergents was reported [39–41, 47, 64, 65].

**Tests for galactolipase activity**

For determination of galactolipase activity in the enzyme preparation isolated from chloroplasts or leaf extracts, pure galactolipids — MGDG or DGDG — were used as substrates [66]; alternatively, subchloroplast particles can be applied as a source of galactolipids [41, 52, 53, 57, 58]. The reaction of galactolipase can be followed also by measurement of FFA release in chloroplasts of chilled leaves [52, 53, 60] or aged chloroplasts [67]. Beside the determination of total content of FFA in chloroplast fraction by standard GLC methods, a simple and rapid colorimetric procedure with diphenylcarbazide was elaborated [68]. Measurements of decrease of total acyl lipid content in chloroplasts or leaves may be also applied as indicator of galactolipase activity in chilled or post-chilling rewarmed plants.

## GALACTOLIPASE IN THE CHILLING-SENSITIVE AND CHILLING-RESISTANT PLANTS

Composition of acyl lipids in chloroplast membranes from CS and CR plants does not differ (Table 1). However, galactolipase activity in the CS plants: bean [38, 43, 49, 64], potato [47] or cowpea [44] is significantly higher than in CR plants such as cabbage, spinach or sugar beet [38, 51]. In the extract from spinach leaves galactolipase activity towards MGDG and DGDG constituted 4% and 15% [48] of that found in bean leaves [38]. For comparative data on galactolipase activity in several CS and CR plants see [51].

The differences in galactolipase activity in CS and CR plants and the involvement of this enzyme in chilling stress become evident if one considers the effect of FFA, released by galactolipase from membrane lipids on photochemical activity.

In contrast to CR plants such as lettuce and spinach, chilling stress applied to detached leaves of CS plants (bean, cucumber, tomato) results in impairment of their photochemical activities (Fig. 1). This experiment also indicates that the assay of Hill reaction [56] or oxygen evolution [72] may serve as the useful markers of chilling sensitivity.

Figure 2 illustrates the relation between the pronounced release of FFA in chloroplasts of CS bean and tomato during cold treatment of the leaves with an almost constant level of FFA in CR spinach. The level of FFA in chloroplasts is associated in chilling stress with oxygen evolution. Thus the CS and CR plants differ significantly with respect to the amount of FFA released and inactivation of oxygen evolution (Fig. 3), attributed to a more active galactolipase in CS plants [67].

**Table 1. Acyl lipid composition of chloroplast membranes in CS and CR plants**

| Lipid species | CS | CR | |
|---|---|---|---|
| | Bean | Spinach | Wheat |
| | | (μmol/ mg chlorophyll) | |
| MGDG | 1.84 | 1.59 | 1.44 |
| DGDG | 0.90 | 0.77 | 0.97 |
| PG | 0.22 | 0.22 | 0.29 |
| SQDG | 0.51 | 0.13 | 0.24 |
| PC | 0.10 | 0.17 | 0.08 |
| References | [43] | [69] | [42] |

In view of these differences in galactolipase activities between CS and CR plants it is surprising that in some CS plants : members of *Cucurbitaceae* and also in soybean galactolipase activity [38, 51] could not be detected at the normal growth temperature. The enzyme becomes active when chilling stress appears [60]. In CR plants: cabbage [38, 51], sugar beet, turnip [38], *P. quandragularis, P. edulis* and pea [51] galactolipase might also exist in a latent form but remains inactive during chilling stress although it is activated during senescense. This suggests different regulation mechanisms. The failure to detect galactolipase activity in some CS plants might be due to the presence of cytoplasmic inhibitors such as were demonstrated for phospholipase D in spinach [70] and cabbage [71] or those observed during isolation of bean galactolipase [41].

For confirmation of our suggestion on the role of galactolipase in chilling stress due to accumulation of FFA and lowering of photochemical activity we have extended our studies to closely genetically related species of tomato [52], maize [53], cucumber [60] and potato [58]. Figure 4 presents the results obtained with the species of *Licopersicon:* domestic *L. aesculentum* and the *L. hirsutum* and *L. peruvianum* ecotypes growing at dif-

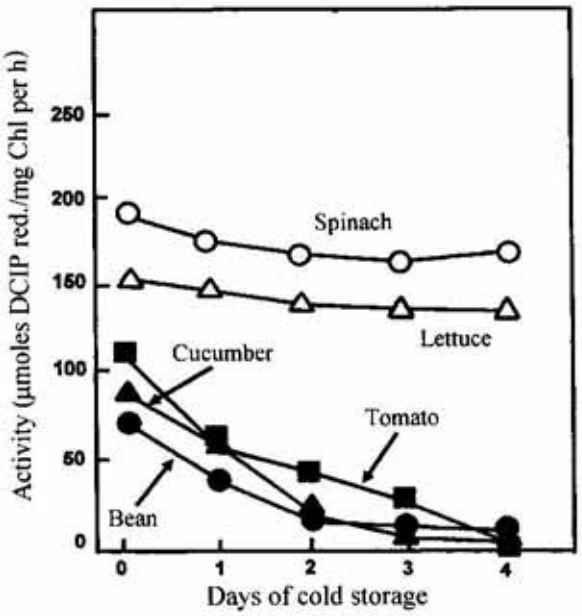



**Figure 1. Hill reaction activity in the cold (0°C to 4°C) and dark stored leaves [56].**

CS plants: bean, cucumber, tomato. CT: spinach, lettuce. DCIP, 2,6-dichlorophenolindophenol.

ferent altitudes. The highest galactolipase activity was found in chloroplasts of domestic *Licopersicon* and successively lower in *L. hirsutum* (700 m), *L. hirsutum* (3100 m) and *L. peruvianum* consistently with the decreasing order of chilling sensitivity, increasing accumulation of FFA and decreased Hill reaction [52]. The same relation was demonstrated between galactolipase activity, FFA

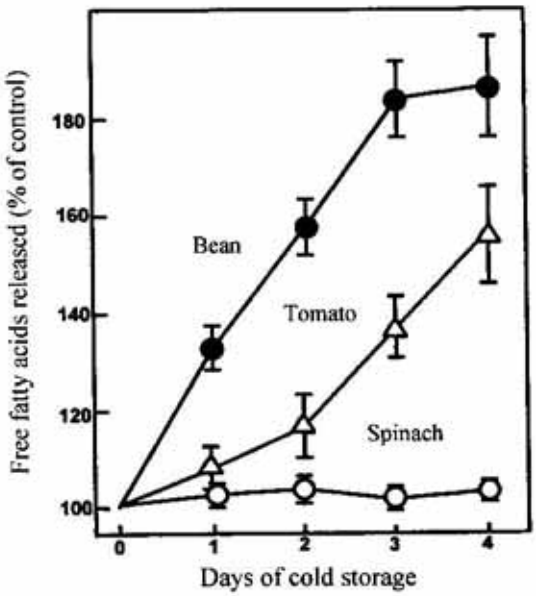



**Figure 2. Accumulation of FFA in chloroplasts during cold (0°C to 4°C) and dark treatment of bean, tomato and spinach leaves [57].**

level and oxygen evolution in the leaves of CS maize line F7 RpIII and CT line S72 [53].

Interesting variation of galactolipase activity was noted within *Cucurbitaceae*, since in members of this CS family the activity of the enzyme could not be detected either in leaf extracts [38], leaf segments [73] or the enzyme preparation isolated from chloroplasts [51, 60], but the enzyme was found to be present in cucumber leaves. The extent of FFA accumulation and inactivation of oxygen evolution in chilled cucumber leaves was typical for CS plant (Fig. 5A, B).

In CS plants accumulation of FFA in chloroplasts during chilling stress preceeds inactivation of oxygen evolution by one day.

Essential differences in galactolipase activ-

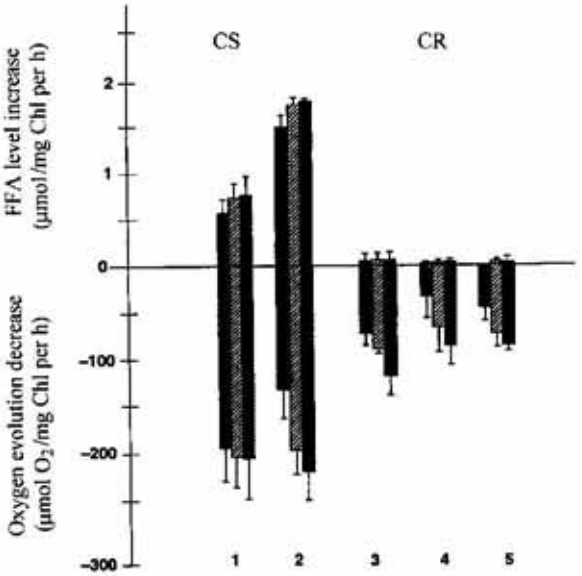
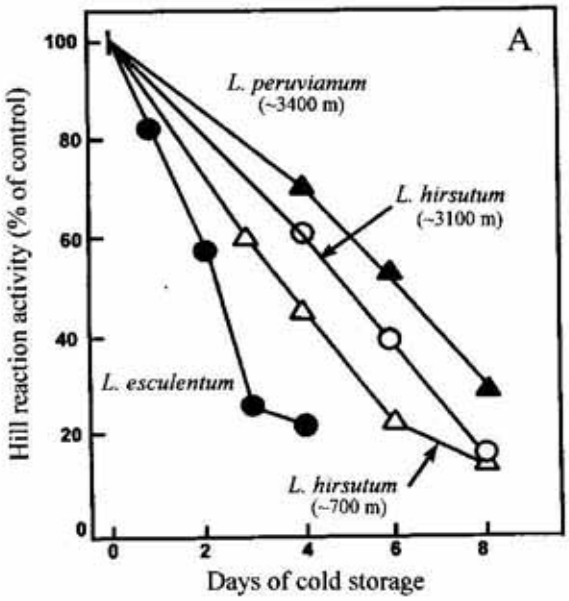


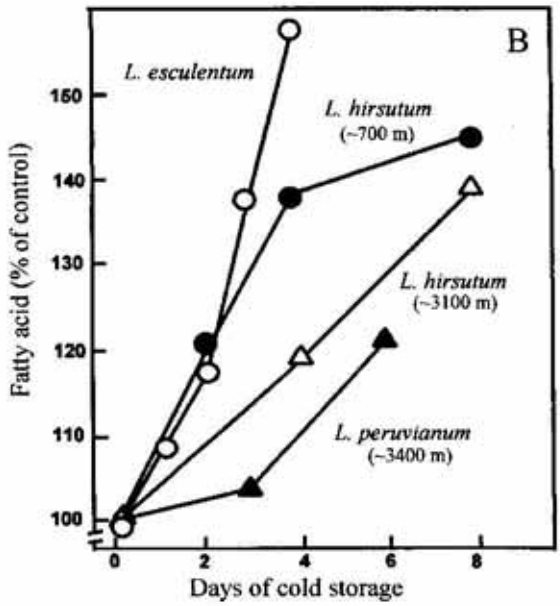

**Figure 3. Decrease of oxygen evolution and increase of FFA level during ageing at 30°C of isolated chloroplasts of CS and CR plants [67].**

CS plants: bean (1), maize-line F7 (2), and CR plants: maize line EP1 (3), pea (4) and wheat (5). Incubation time: 20, 40 and 60 min denoted by sequential bars from left to right.

ity were evidenced in the preparations isolated from chloroplasts of CT tolerant potato species *Solanum ajanhuirrand* and *S. chaucha*, as well as CS *S. toralapanum and S. tuberosum*. Galactolipase activity expressed in μmols of FFA released per min per mg protein amounted to nil and 0.09, respectively, for CT potato species and 0.51 and 0.64, respectively, for CS species [58]. Upon chilling of the leaves for 6 days the ratio of

FFA content in chloroplasts from chilled and non chilled leaves was found to be 3.6 to 4.8 for CS species and 1.0 to 1.6 for CT species. These data are consistent with the previously found correlation between galactolipase activity and chilling sensitivity.




Since the levels of acyl lipids in CS and CR plants are practically the same (Table 1) and FFA level is increased on chilling only in CS plants the existence of two separate FFA pools could be hypothesized: an original one and the secondary pool generated during chilling or ageing of chloroplasts [60].

The original content of FFA differs among closely related cultivars or inbred lines by the factors 2, 3, 4, and 6 in tomato [52], cucumber [60], maize and red pepper [59], respectively. In CS maize lines and red pepper cutivars [59] the original high level of FFA in chloroplasts remained constant during few days of chilling and this content had no effect on oxygen evolution, a characteristic marker of chilling sensitivity [56, 72]. On the other hand, chilling of the leaves [52, 53, 57–60] and ageing of chloroplasts [67] of CS species resulted in an increase of FFA level of secondary pool which was associated with lowering of oxygen evolution. The size of this pool seems to be related to galactolipase activity in these plants and reflects their chilling sensitivity.

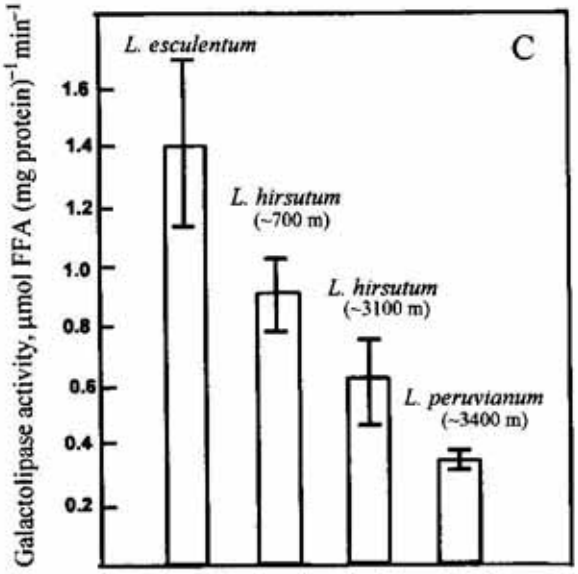



**Figure 4. Effect of cold treatment of detached leaves of domestic and wild tomatoes (*Licopersicon*) with various chilling tolerance [52].**

(A) Decrease in Hill reaction; (B) accumulation of FFA in chloroplasts, and (C) galactolipase activity in isolated preparation from control leaves.

Differences between CS and CR plants do not only involve galactolipase activity and the response to chilling but also may concern

**Table 2. Composition of acyl lipids and proportion of high-melting-point PG species in the leaves of CS and CT maize.**

Characteristics of maize lines are given in [59, 74–76]. Fourteen days old seedlings were grown in the controlled conditions at 22°C/20°C day/night under 16 h photoperiod with irradiance of 80 μmol × $m^2$ per $s^{-1}$ (V. Sączyńska, E. Miśkiewicz & Z. Kaniuga, unpublished).

| CS and CT inbred lines | | MGDG | DGDG | SQDG | PG | HMFA in PG |
|---|---|---|---|---|---|---|
| | | (μmol/mg chlorophyll) | | | | (mol %) |
| CS | CM7 | 2.18 | 1.64 | 0.30 | 0.52 | 59.9 |
| | Co151 | 2.30 | 1.55 | 0.37 | 0.49 | 58.1 |
| CT | S215 | 2.30 | 1.49 | 0.34 | 0.55 | 58.7 |
| | EP1 | 2.32 | 1.54 | 0.33 | 0.51 | 59.7 |

enzyme properties in these two groups of plants [65]. The different pH-dependence curve and different response to detergents of galactolipase from CS and CR plants might indicate heterogeneity of the enzyme [65]. As shown in Fig. 6 the optimum pH of galactolipase activity in CS maize line F7 was 5.5 while in CT EP1 line the activity is low and does not change within the range from 5.0 to 8.5. In general in CS plants exemplified by CS bean detergents changed the pH optimum towards alkaline values. No effect of detergents was noted on the activity of CR pea even some stimulation by Tween 20 was evidenced [65].

## GALACTOLIPASE ACTIVITY BUT NOT DISATURATED PG CONTENT IS RESPONSIBLE FOR CHILLING SENSITIVITY IN MAIZE

Composition of acyl lipids in the leaves of CS inbred lines of maize CM7 and Co151 is not different from those of CT lines S215 and EP1 (Table 2). The relative proportion within acyl lipids, as well as their contents in individual inbred lines are surprisingly similar. This may suggest that the substrates for galactolipase in all inbred lines are equally available. In addition, the content of HMFA-PG is also equal. Therefore, according to the

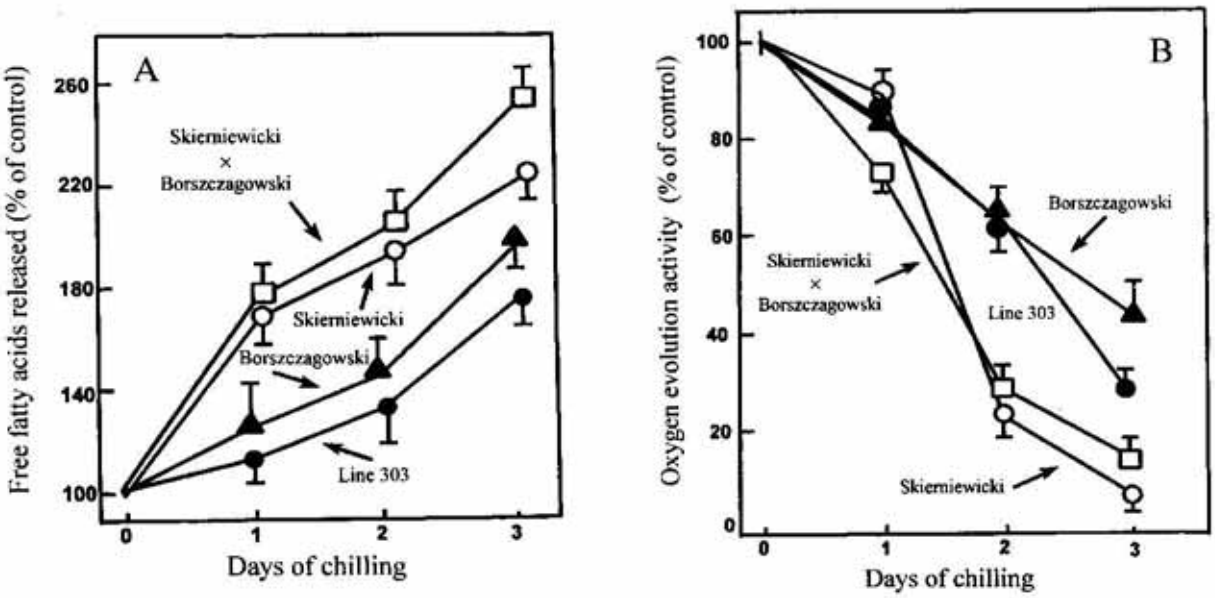



**Figure 5. Accumulation of FFA (A) and oxygen evolution (B) in chloroplasts of CS and CT cucumber species during cold (4.5°C) storage of detached leaves [60].**

CS species: Skierniewicki and cross Skierniewicki × Borszczagowski; CT species: Borszczagowski and line 303.

"HMFA-PG" hypothesis, no differences in chilling sensitivity should be expected between these two groups.

During chilling of maize seedlings at 5°C in the dark for 4 and 6 days the total acyl lipid content decreased in CS and CT inbred lines approximately by about 15.5% and 12.5%, respectively. After 6 days of chilling under these conditions about 34% and 26% decrease of MGDG in CS and CT inbred lines, respectively, was observed. Thus, differences between CS and CT inbred lines were too small for the differentiation of their chilling sensitivity.

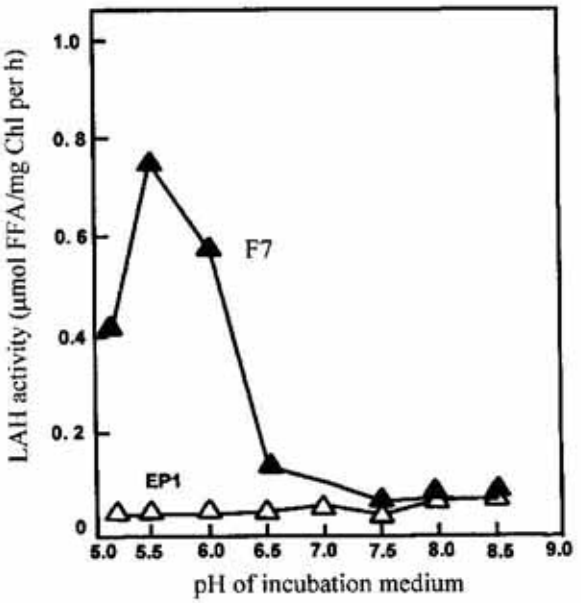



**Figure 6. Effect of pH on galactolipase (LAH) activity in maize chloroplasts of CS line F7 and CT line EP1 [65].**

When maize seedlings were returned for 4 days to the previous growth conditions, characteristic damage to the leaves of CS plants with visible lesions such as necrotic areas, dead leaf tips or dead leaf margins were observed, differentiating CS from CT inbred lines. These morphological changes were accompanied by more extensive degradation of total acyl lipid content in CS maize inbred lines by about 53% than in CT plants by about 20% of the total content of acyl lipids in the leaves during post-chilling rewarming. Degradation of individual acyl lipids was also proportionally more extensive in CS than in CT inbred lines.

## GALACTOLIPASE ACTION AND LIPID PEROXIDATION

Peroxidation of membrane lipids is generally believed to be an important factor in the mechanism of chilling injury, accelerating damage to membrane integrity [7]. The extent of peroxidation in thylakoids of CS bean and CR pea depends on the level of FFA main substrates for peroxidation [77], which was higher in bean than in pea thylakoids. The FFA fraction in chloroplasts contains a large proportion of 18:2 and 18:3 acids (up to 70–80% of the total FFA [52, 60, 78]), which are particularly accessible to peroxidation.

Involvement of oxygen reactive species in the initiation of peroxidation is commonly accepted, but recent data concerning this problem are not consistent. Thus, although chilling of leaf slices of cucumber at moderate light did stimulate $O_2^-$ -production in chloroplasts [79], it did not increase peroxidation either of membrane lipids in cucumber [63, 80] and oleander growing at 45°C [80] or on addition of methyl viologen [63], a well known effector of $O_2^-$ -production, to cucumber leaf fragments. In spite of the chilling-induced increase of $O_2^-$ -production in CS plants [79], this effect was not reflected in the extent of peroxidation of membrane lipids in cucumber and spinach leaf slices upon chilling [80]. The lack of relation between peroxidation of lipids and response to chilling of CR pea [63] and spinach [80] species and that in CS cucumber [63, 80] was attributed to the presence of some endogenous mechanism for removal of toxic oxygen species prior to lipid peroxidation in CR plants [63]. Alternatively, it was suggested [80] that $O_2^-$ -produced in chloroplasts of CR plants is more efficiently dismutated to $H_2O_2$ and oxygen by superoxide dismutase, and, therefore, peroxidation is limited. However, in these experiments no significant differences in the enzyme activity were found either between CS cucumber and oleander grown at 45°C and CR spinach and oleander grown at 20°C, or on incubation of their slices at chilling temperature [80].

Another argument against generalization of the peroxidation effect in CS and CR plants deals with the effect of methyl viologen as an

effector of $O_2^-$ -production. In pea leaf segments ethane production was observed neither in the cold, nor in the light for 6 h unless they were pretreated with methyl viologen. After this period ethane was produced at a rate which was equal in the chilled and irradiated CS cucumber leaf segments [63]. The question arises why the level of $O_2^-$ -inceased by methyl viologen did not stimulate production of ethane in cucumber, while it did in pea? On the other hand, Hodgson & Raison [80] did not observe any stimulation of peroxidation by methyl viologen in chilled spinach leaf slices and even they noticed inhibition of peroxidation under these conditions.

There is no agreement either with respect to the effect of temperature on peroxidation. At low temperature (5°C) peroxidation was almost 2–4-fold more effective than at 25°C in cucumber leaf segments [63, 81], however, malondialdehyde formation in cucumber, spinach and oleander leaf slices at 4°C was only by 15% lower than in the slices incubated for the same time at 25°C [80].

All these discrepancies seem to indicate the involvement in lipid peroxidation in CS and CT species of some other factors, which would be responsible for the differences in efficiency of peroxidation in these plants. Some of these discrepancies may be explained by different level of FFA in chloroplasts of CS and CT plants due to the action of galactolipase.

## QUESTIONS AND CONCLUSIONS

Although the content of acyl lipids and their composition in chloroplasts of CS and CR plants do not differ essentially, it could be expected that the activity of galactolipase might also be similar. However, this is not the case. In addition, the enzyme in CS plants is activated during chilling stress. Is it an accidental or characteristic property of the enzyme in CS plants, related to their chilling sensitivity?

The second question refers to the mechanism by which the apparently inactive enzyme appears to become active during chilling stress. Therefore, better understanding of the action and control of galactolipase activity *in vivo* and during chilling is needed. An unexplained difference in the chilling response in plants with the same level of disaturated-PG contradicts the validity of generalization of the essential role of this PG molecular species in chilling sensitivity.

The results obtained with a very large number of plant species, including genetically close ones, imply an important role of galactolipase in chilling stress. Univocal proof of participation of galactolipase in the chilling response could be obtained by genetically engineered reduction of the enzyme of CS plants.

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